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13. ABSTRACT (Maximum 200 Words)

High levels of *Wilms' Tumor 1 (WT1)* mRNA in breast tumors have been linked with poor prognosis for breast cancer patients. However, the function of WT1 protein in breast cancer was not known. We demonstrated that WT1 protein is vital to the proliferation of breast cancer cells since downregulation of WT1 protein expression led to breast cancer growth inhibition. We also demonstrated that the WT1 protein expression is increased by 17☐-estradiol, but inhibited by tamoxifen or all-*trans* retinoic acid. We have expanded our studies and found that two other poor prognostic indicators of breast cancer patients: HER2/neu and Insulin-like Growth Factor-I use the Akt pathway to increase WT1 expression. WT1 has been shown to undergo two splicing events, which result in four different isoforms. We have preliminary data indicating that the two isoforms "A" and "D" are stimulate the proliferation of MCF-7 breast cancer cells. However, the WT1 isoforms do not appear to modulate the sensitivities of MCF-7 cells to doxorubicin and taxol. We plan to determine the mechanisms and the isoforms by which WT1 deregulates breast cancer cell proliferation.

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Table of Contents

INTRODUCTION

The Wilms' Tumor 1 (WT1) protein and mRNA is expressed in human breast tumors and breast cancer cell lines. High levels of WT1 mRNA have been correlated with poor prognosis for breast cancer patients, and expression of WT1 protein was correlated with breast cancer cell proliferation. One aim of this project will be to determine if the WT1 protein contributes to breast tumor progression by deregulating cell proliferation and apoptosis. The deregulation of proliferation and survival pathways has been associated with chemoresistance in many tumors. Therefore, it is hypothesized that WT1 regulates chemoresistance in breast cancer cells. WT1 has been shown to undergo two splicing events, which result in four different isoforms. These isoforms are able to bind to different DNA promoter elements and different protein partners. This project seeks to determine the mechanisms and the isoforms by which WT1 deregulates breast cancer cell proliferation and apoptosis.

BODY

Specific Aim 1: To determine whether WT1 overexpression increases the proliferation and survival of breast cancer cells in cell culture models

In the second year of funding, the Principal Investigator (PI) successfully transfected MCF-7 breast cancer cells with the "A" and "D" isoforms of the WT1 gene. Overexpression of the gene was confirmed by Western blot and RT-PCR. Unlike the first year, this time the transfection remained stable for up to at least 6 months. Cell counting and the CellTiter 96 Aqueous nonradioactive proliferation (MTS) assay were used to determine the proliferative rates of these transfectants. Compared to parental cells, both the "A" and the "D" isoforms increase MCF-7 cell proliferation by 150%. We plan to perform flow cytometry to determine in which phase(s) of the cell cycle WT1 is involved. We are currently transfecting the other two WT1 isoforms "B" and "C" into MCF-7 cells.

Specific Aim 2: To determine whether WT1 overexpression increases breast tumor growth in animal models

MCF-7 wild type cells and MCF-7 transfectants (vector control, WT1 isoform "A", WT1 isoform "D") were implanted into the mammary fat pad of nude mice that had 0.72 mg of 17β-estradiol pellets. Four weeks later, tumors were found from mice implanted with the control wild type and vector cells. Six out of nine mice implanted with MCF-7wild type cells form tumors, and eight out of eight mice implanted with MCF-7/vector cells form tumors. However, no tumor was found in mice implanted with the MCF-7 cells stably transfected with the WT1 "A" isoform or the WT1 "D" isoform. We will repeat this experiment to confirm our observations. Furthermore, once we obtain the other WT1 isoform transfectants ("B" and "C"), we will also implant them into nude mice.

Specific Aim 3: To determine whether WT1 regulates chemoresistance in breast cancer cells

MTS assay was used to compare the chemosensitivities of WT1 transfectants with control cells. No difference is observed between the doxorubicin sensitivity of WT1 "A" or WT1 "D" isoform and the control cells. Similarly, no difference is observed between the taxol sensitivity of WT1 "A" or WT1 "D" isoform and the control cells. We plan to compare the sensitivities of these transfectants to estrogen and tamoxifen. Furthermore, once we obtain the other WT1 isoform transfectants ("B" and "C"), we will determine their sensitivities towards chemotherapy and hormonal therapy.

3

Since high levels of WT1 expression had been associated with the more aggressive phenotypes of breast cancer, we believe it is important to determine what factors regulate WT1 expression. We had expanded on our preliminary studies from last year and confirmed that the *HER2/neu* oncogene uses Akt to increase the expression of WT1 protein. Moreover, WT1 mediates proliferative and anti-apoptotic functions in HER2/neu-overexpressing breast cancer cells. We had also determined the effects of Insulin-like Growth Factor I (IGF-1) and Epidermal Growth Factor (EGF), which had been inversely associated with breast cancer patients prognosis, on WT1 expression. We found that IGF-1 uses Akt to stimulate WT1 transcription. However, EGF has no effect on WT1 expression.

KEY ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.
- Data indicates that the *HER2/neu* oncogene uses the Akt pathway to increase the expression of WT1 protein. WT1 plays a vital role in mediating proliferative and anti-apoptotic functions in HER2/*neu*-overexpressing breast cancer breast cancer cells.
- Data indicates that IGF-1 uses Akt to stimulate WT1 transcription.
- Data indicates that EGF has no effect on WT1 expression.

REPORTABLE OUTCOMES:
Manuscripts
1. M. Tuna and A. M. Tari. Wilms' Tumor 1 (WT1) protein mediates proliferative and anti-apoptotic functions in HER2/*neu*-overexpressing breast cancer cells. Submitted. (See attached)
2. M. Tuna, A. Sanguino and A. M. Tari. Insulin-like Growth Factor I, but not epidermal growth factor, stimulates Wilms' Tumor 1 (WT1) expression. Manuscript in preparation.

CONCLUSIONS:
We are surprised that the two WT1 isoforms "A" and "D", which have been shown to bind to different partnering proteins and different DNA sequences, appear to behave quite similarly *in vitro* and *in vivo* in the MCF-7 breast cancer cell background. Nonetheless, the project is proceeding as planned, and we will continue investigating our specific aims.

4

Wilms' Tumor 1 (WT1) protein mediates proliferative and anti-apoptotic functions in

HER2/*neu*-overexpressing breast cancer cells

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Running title: Role of WT1 in HER2-overexpressing cells

1

ABSTRACT

High levels of the Wilms' Tumor 1 (*WT1*) protein and mRNA had been associated with aggressive phenotypes of breast tumors. Here we report the novel finding that the *HER2/neu* oncogene increases WT1 expression. Approximately 3-fold higher levels of WT1 protein were observed in MCF-7 breast cancer cells transfected with the *HER2/neu* oncogene than in parental MCF-7 cells. Conversely, inhibition of HER2/*neu* with the anti-HER2/*neu* trastuzumab (Herceptin™) antibody decreased WT1 protein levels in HER2/*neu*-overexpressing BT-474 and SKBr3 cells. We also found that HER2/*neu* uses Akt to regulate WT1 levels since inhibition of Akt reduced WT1 levels. Decreased expression of WT1 protein led to cell cycle arrest at the G1/S phase and increased apoptosis in HER2/*neu*-overexpressing cells, which is correlated with decreased cyclin D1 and Bcl-2 levels. Thus, WT1 protein plays a vital role in mediating the proliferative and anti-apoptotic signals in HER2/*neu*-overexpressing breast cancer cells.

INTRODUCTION

The Wilms' tumor 1 (*WT1*) gene was originally identified as a tumor suppressor gene responsible for Wilms' tumor. In addition to germ-like mutations, somatic mutations of WT1 as well as loss of heterozygosity at the 11p13 locus harboring WT1 have been reported in sporadic Wilms' tumors. WT1 is a zinc finger-containing nuclear protein that acts as a transcription factor by binding to CG-rich sequences and TCC-rich sequences on promoters of target genes. [1] High levels of the wild type *WT1* mRNA had been found in leukemias, [2,3] lung tumors [4] and breast tumors. [5,6] Patients with high *WT1* mRNA levels in their breast tumors were found to have a lower five-year disease-free survival rate than patients whose breast tumors expressed low *WT1* mRNA levels. [6] WT1 expression had been associated with the more biologically aggressive phenotypes of breast tumors, such as Estrogen Receptor (ER) negativity, tumors > 2 cm and with disease recurrence. [6,7] These data strongly suggest that WT1 expression is vital to breast cancer, perhaps especially in the aggressive and poor prognosis phenotypes. However, it is not known what factors in the biologically aggressive breast tumors cause higher WT1 expression levels, and the functions of WT1 protein in such tumors.

Amplification of the *HER2/neu* oncogene is found in 30% of human mammary cancers and is associated with poorer prognosis in breast cancer patients. [8,9] HER2/neu-overexpressing breast tumors are of the aggressive phenotype and are likely to be ER negative. Thus we speculate that HER2/*neu* may be one factor that increases WT1 levels. Here we show that HER2/*neu* uses Akt to stimulate WT1 expression, and that WT1 protein mediates proliferative and anti-apoptotic functions in HER2/*neu*-overexpressing breast cancer cells, possibly via cyclin D1 and Bcl-2 proteins.

3

MATERIALS AND METHODS

Cell lines

BT-474, SKBr3, and parental MCF-7 (MCF-7/WT) cells were obtained from American Type Cell Culture (Manassas, VA, USA). MCF-7 cells transfected with *HER2/neu* gene (MCF-7/ HER2) were kindly provided by Dr Mien-Chie Hung (Houston, TX, USA). BT-474, SKB3, and MCF-7/WT cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 5% heat-inactivated fetal bovine serum (FBS). MCF-7/HER2 cells were cultured in DMEM/F12 medium supplemented with 5% FBS and 500 µg/mL Geneticin (G418).

Reagents

Monoclonal antibodies specific for WT1 (6F-H2) and cyclin D1 were purchased from DAKO (Carpinteria, CA) and NeoMarkers (Fremont, CA, USA), respectively. Antibodies specific for HER2/neu and Grb2 were obtained from Oncogene (Cambridge, MA, USA), and BD Transduction Laboratory (San Diego, CA, USA), respectively. Phosphorylated antibodies specific for HER2/*neu* (Tyr^{1248}), and GSK-3β (Ser^9) were purchased from Cell Signaling Technology (Beverley, MA, USA). Anti-mouse and anti-rabbit secondary antibodies were obtained from Amersham Life Sciences (Cleveland, OH, USA). Trastuzumab (Herceptin^{TM}) was kindly provided by Genentech (San Fransisco, CA, USA). The Akt inhibitor (IL-6-hydroxymethyl-chiro-inositol-2®-20-methyl-3-O-ocadecylcarbomate), purchased from Calbiochem (San Diego, CA, USA), was dissolved in DMSO at a concentration of 10 mM and stored at -20°C. Trastuzumab was stored at –20°C at a concentration of 40 mg/mL.

Liposome-incorporated oligos

P-ethoxy oligos were purchased from Oligos Etc., Inc. (Wilsonville, OR, USA). The sequence of the WT1 antisense and the control oligos [10] were as follows: WT1 antisense, 5'-GTC GGA GCC CAT TTG CTG-3' and control oligo, 5'-TCG CGA CTG GAT CCT GCC CG -3'. Briefly, P-ethoxy oligos were mixed with dioleoylphosphocholine in the presence of tertiary butanol, frozen in a dry ice/acetone bath, lyophilized and prepared as described. [11]

Western Blot

Cell lysates were obtained from untreated cells or from cells treated with trastuzumab, liposomal oligos, or Akt inhibitor. Protein concentration was determined by using the Bio-Rad DC reagent kit (Bio-Rad Laboratories, Hercules, CA, USA). Proteins (50 µg) were electrophoresed on 10% SDS polyacrylamide gels and transferred to nitrocellulose membranes. Protein bands were visualized by enhanced chemiluminescence (Kirkegaar & Perry Laboratories, Gaithersburg, MD). Images were scanned and quantified by using an Alpha Innotech densitometer using the Alpha Imager application program (Alpha Innotech, San Leandro, CA, USA).

Flow cytometric analysis

Breast cancer cells were incubated with 14 µM liposomal oligos for 5 days. Cells were trypsinized and approximately 1×10^6 cells were collected by centrifugation at 1500 rpm for 5 min, washed, and resuspended in 1 mL PBS. The cell suspension was added to 1 mL cold 70% ethanol and incubated overnight at -20°C. Cells were centrifuged at 1500 rpm for 10 min at 4°C, washed in PBS, and the pellet was left loose. Approximately 0.5-1 mL PBS containing RNase (20 µg/mL) and propidium iodide (50 µg/mL) was added to each cell pellet, followed by 20 min

of incubation at room temperature. Flow cytometric analysis was performed using a Coulter Epics Profile 488 laser.

RNA extraction and RT-PCR

Total RNA was prepared from SKBr-3 and BT-474 cells using 1 mL TRIzol Reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. The pellet of RNA was dissolved in diethylpirocarbinate (DEPC)-treated-water and quantified by spectrophotometry at 260 nm. cDNA was created with Superscript II according to the manufacturer's protocol (Gibco BRL). All PCR reactions carried out with 5 μL cDNA, 0.2 mM dNTPs, 2.5 μM of each primer, 10 mM Tris-HCl (pH 8.4, 50 mM KCl, 0.01% gelatin, 2.0 mM MgCl$_2$), and 2.5 U Taq DNA polymerase. The sequences of the cyclin D1 primers: forward, 5'-CTG GAG CCC GTG AAA AGA GC-3' and reverse, 5-'CTG GAG GAA GCG TGT GAG G-3'. The sequences of the Bcl-2 primers were: forward, 5'- TGC ACC TGA CGC CCT TCA C-3' and reverse, 5'-AGA CAG CCA GGA GAA ATC AAA CAG-3'. The sequences of the β-actin primers were: forward, 5'-GTC ACC AAC TGG GAC GAC ATG-3' and reverse, 5'-GAC AGC ACT GTG TTG GCG TAC-3'. The PCR conditions for cyclin D1 were as follows: 94°C for 3 min, 5 cycles of 94°C for 30 sec and 72°C for 45 sec, 25 cycles of 94°C for 30 sec, 56°C for 1 min and 72°C for 2 min, followed by 7 min at 72°C. Identical PCR conditions were used for Bcl-2 except that 30 cycles were used. The PCR conditions for β-actin were 94°C for 5 min, and 30 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 45 sec, followed by 7 min at 72°C. PCR products were subjected to electrophoresis on 1% agarose gels and the reaction products were visualized with ethidium bromide and photographed under UV transillumination.

RESULTS

HER2/neu increases WT1 protein expression in breast cancer cells

Approximately three-fold higher levels of WT1 protein were observed in MCF-7 cells transfected with the *HER2/neu* gene (MCF-7/*HER2*) than in parental MCF-7 (MCF-7/WT) cells (Figure 1A). To further prove that HER2/neu increases WT1 protein expression, trastuzumab (Herceptin™) was used to inhibit HER2/neu function. SKBr3 and BT-474 breast cancer cell lines, which are known to have *HER2/neu* gene amplification, [12] were incubated with 0.5 μM and 0.1 μM trastuzumab, respectively, for 5 days. Incubation of breast cancer cells with trastuzumab led to decreased HER2/*neu* function as indicated by decreased phosphorylation of the Tyr^{1248} residue in the HER2/*neu* protein (Figure 1B). Blocking *HER2/neu* led to decreased WT1 protein levels by 51% and 43% in SKBr3 and BT-474 cells, respectively (Figure 1B). These data indicate that HER2/*neu* increases WT1 protein levels.

HER2/neu uses Akt to regulate WT1 protein expression

We have previously shown that Akt is an important downstream signaling protein of HER2/*neu*. [13-15] To determine whether HER2/*neu* uses Akt to regulate WT1 expression, SKBr3 and BT-474 cells were incubated with 10 and 20 μM Akt inhibitor, IL-6-hydroxymethyl-chiro-inositol-2®-20-methyl-3-O-ocadecylcarbomate, respectively. As expected, the Akt inhibitor decreased the phosphorylation of the Ser^9 residue of GSK-3β, a downstream protein of Akt. The Akt inhibitor decreased WT1 protein expression in a time-dependent manner in SKBr3 and BT-474 cells (Figure 2). These data indicate that Akt is vital for the increased WT1 expression in HER2/*neu*-overexpressing cells.

7

Downregulation of WT1 protein expression induces cell cycle arrest and apoptosis in HER2/neu-overexpressing breast cancer cells

Previously we demonstrated that WT1 protein is vital for the growth of BT-474 and SKBr3 cells since downregulation of WT1 protein expression by liposome-incorporated WT1 (L-WT1) antisense oligos led to growth inhibition in both cell lines [10]. However, it is not known in which phase of the cell cycle WT1 is involved. Flow cytometric analysis was used. BT-474 cells were incubated with L-WT1 antisense oligos and liposome-incorporated control (L-control) oligos for 5 days. L-WT1 antisense oligos increased the percentage of cells in the G1 phase by 16%, and decreased the percentage of cells in the S phase by 15%. L-WT1 antisense oligos also increased the percentage of apoptotic cells, as indicated by the sub-G1 phase, from 2.2 to 37.8% (Figure 3). However, L-WT1 antisense oligos did not affect the G2/M phase. Under the same conditions, L-control oligos did not affect the cell cycle distribution (Figure 3). Similar effects were observed in SKBr3 cells. L-WT1 antisense oligos increased the percentage of SKBr3 cells in the G1 phase by 10%, but did not affect the G2/M phase (data not shown). L-WT1 antisense oligos also increased the percentage of SKBr3 cells undergoing apoptosis from 5.9 to 10.7% (data not shown). These data indicate that WT1 mediates proliferative and anti-apoptotic functions in HER2/neu-overexpressing breast cancer cells.

WT1 protein regulates cyclin D1 and Bcl-2 expression at the transcription level

We then determined which cell cycling and apoptotic proteins may be regulated by WT1. Western blot was performed on BT-474 cells incubated with L-WT1 antisense oligos, L-control oligos and empty liposomes. L-WT1 antisense oligos inhibited WT1 protein expression

by 50% (Figure 4A), whereas L-control oligos and empty liposomes did not affect WT1 protein expression (Figure 4A). Downregulation of WT1 protein expression led to decreased cyclin D1 and Bcl-2 protein levels (Figure 4A). WT1 has been shown to regulate Bcl-2 transcriptionally [16-18]. We speculate that WT1 also regulates cyclin D1 at the transcriptional level, since the cyclin D1 promoter contains CG-rich elements that may be potential WT1 consensus sites. Untreated and treated BT-474 cells were subjected to RT-PCR. L-WT1 antisense oligos decreased *cyclin D1* and *Bcl-2* mRNA levels (Figure 4B). Under identical conditions, L-control oligos and empty liposomes did not have any effects on cyclin D1 and Bcl-2 levels (Figure 4). These data indicate that WT1 regulates cyclin D1 and Bcl-2 transcriptionally in HER2/*neu*-overexpressing cells.

DISCUSSION

Our data indicate that the HER2/*neu* oncogene increases WT1 protein expression in breast cancer cells. MCF-7 cells stably transfected with the *HER2/neu* oncogene had higher WT1 protein levels than parental MCF-7 cells. Inhibition of HER2/*neu* by the anti-HER2/*neu* trastuzumab antibody led to decreased WT1 expression in HER2/*neu*-overexpressing BT-474 and SKBr3 cells. Our data are in agreement with previous studies that had associated high levels of *WT1* mRNA and protein expression with the more aggressive phenotypes of breast tumors. [6,7] Patients whose breast tumors have the HER2/*neu* amplification are known to have poorer prognosis and outcome than patients whose tumors do not have HER2/*neu* amplification. [8,9]

WT1 represses or activates transcription from the target gene's promoter. WT1 plays a vital role in HER2/*neu*-overexpressing breast cancer cells. WT1 mediates proliferative and anti-apopototic functions in such cells, at least in part, by regulating the levels of cyclin D1 and Bcl-2. Cyclin D1 is known to be a downstream target of HER2/*neu*. In transgenic mice studies, cyclin D1 has been shown to be vital for *HER2/neu*-induced mammary tumorigenesis. [1,19] Previously Lenferink et al. [19] reported that HER2/neu regulates cyclin D1 transcriptionally and translationally. Here we show that WT1 is another mechanism by which HER2/*neu* regulates cyclin D1 levels. Downregulation of WT1 protein expression by L-WT1 oligos led to decreased *cyclin D1* mRNA levels. We speculate that WT1 upregulates cyclin D1 at the transcriptional level because the cyclin D1 promoter contains CG-rich elements that may be potential WT1 consensus sequences. One possible mechanism by which WT1 prevents cells from undergoing apoptosis is by upregulating anti-apoptotic genes such as Bcl-2. WT1 has been shown to regulate

the Bcl-2 protooncogene by regulating the bcl-2 promoter through a high affinity WT1 consensus site. [16-18]

It is known that Akt activates downstream proteins by phosphorylating them at the consensus sequence site (RXRXXS). [20] However, we could not identify such Akt consensus site within the WT1 sequence. We are currently investigating which factors downstream of Akt are involved in the regulation of WT1 expression.

WT1 protein appears to have a growth regulatory role in acute leukemias, and solid tumors, such as breast cancer, gastric cancer, lung cancer, and ovarian cancer since decreased expression of WT1 protein levels led to growth inhibition and apoptosis in these cancers [10, 21-25]. We previously showed that 17β-estradiol increases WT1 protein expression in ER positive breast cancer cells. [10] Here, we report the novel finding that HER2/*neu*, whose expression is often found in ER negative breast cancer cells, increases WT1 levels, and that WT1 protein mediates proliferative and anti-apopotic function in such cells, possibly via cyclin D1 and Bcl-2.

ACKNOWLEDGEMENTS

REFERENCES

1.   Lee S, Haber DA. Wilms Tumor and the WT1 gene. Exp Cell Res 2001;264:74-99.

2.   Inoue K, Sugiyama H, Ogawa H, Nakagawa M, Yamagami T, Miwa H, et al. WT1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. Blood 1994;84:3071-79.

3.   Inoue K, Ogawa H, Sonoda Y, Kimura T, Sakabe H, Oka Y, et al. Aberrant overexpression of the Wilms tumor gene (WT1) in human leukemia. Blood 1997;89:1405-12.

4.   Oji Y, Miyoshi S, Maeda H, Hayashi S, Tamaki H, Nakatsuka S, et al. Overexpression of the Wilms' tumor gene WT1 in de novo lung cancers. Int J Cancer 2002;100:297-303.

5.   Loeb D, Evron E, Patel C, Sharma P, Niranjan B, Buluwela L, et al. Wilms' tumor suppresor gene (WT1) is expressed in primary breast tumors despite tumor-specific promoter methylation. Cancer Res 2001;61:921-25.

6.   Miyoshi Y, Ando A, Egawa C, Taguchi T, Tamaki Y, Tamaki H, et al. High expression of wilms' tumor suppressor gene predicts poor prognosis in breast cancer patients. Clin Cancer Res 2002;8:1167-71.

7.   Silberstein G, Van Horn K, Strickland P, Roberts C, Daniel C. Altered expression of the WT1 wilms tumor suppressor gene in human breast cancer. Proc Natl Acad Sci U S A 1997;94:8132-37.

8.   Slamon D, Clark M, Wong S, Levin W, Ullrich A, McAuire W. Human breast cancer: correlation of relapse and survival with amplification of the Her-2 neu oncogene. Science 1987;235:177-81.

9.   Slamon D, Godolphin W, Jones L, Holt J, Wong S, Keith D, et al. Studies of the HER-2/neu protooncogene in human breast and ovarian cancer. Science 1989;244:707-12.

10.   Zapata-Benavides P, Tuna M, Lopez-Berestein G, Tari AM. Downregulation of Wilms' tumor 1 protein inhibits breast cancer proliferation. Biochem Biophys Res Commun 2002;295:784-90.

11.   Tari AM. Preparation and application of liposome-incorporated antisense oligonucleotides. Methods Enzymol 2000;313:372-88.

12.   Yakes FM, Chinratanalab W, Ritter CA, King W, Seelig S, Arteaga CL. Herceptin-induced inhibition of phosphatidylinositol-3 kinase and Akt is required for antibody-mediated effects on p27, cyclin D1, and antitumor action. Cancer Res 2002;62:4132-41.

13.   Lim S-J, Lopez-Berestein G, Hung M-C, Lupu R, Tari AM. Grb2 downregulation leads to Akt inactivation in heregulin-stimulated and ErbB2-overexpressing breast cancer cells. Oncogene 2000;19:6271-76.

14.   Tari AM, Lim S-J, Hung M-C, Esteva F, Lopez-Berestein G. HER2/neu induces all-trans retinoic acid (ATRA) resistance in breast cancer cells. Oncogene 2002;21:5224-32.

15.   Simeone AM, Li YJ, Broemeling LD, Johnson MM, Tuna M, Tari AM. Cyclooxygenase-2 is essential for HER2/neu to suppress N- (4-hydroxyphenyl)retinamide apoptotic effects in breast cancer cells. Cancer Res 2004;64:1224-8.

16.   Hewitt S, Hamada S, McDonnell T, Rauscher Fr, Saunders G. Regulation of the proto-oncogenes bcl-2 and c-myc by the Wilms' tumor suppressor gene WT1. Cancer Res 1995;55:5386-89.

17. Mayo M, Wang C, Drouin S, Madrid L, Marshall A, Reed J, et al. WT1 modulates apoptosis by transcriptionally upregulating the bcl-2 proto-oncogene. EMBO J 1999;18:3990-4003.

18. Cheema SK, Mishra SK, Rangnekar VM, Tari AM, Kumar R, Lopez-Berestein G. Par-4 transcriptionally regulates Bcl-2 through a WT1-binding site on the bcl-2 promoter. J Biol Chem 2003;278:19995-20005.

19. Lenferink AE, Busse D, Flanagan WM, Yakes FM, Arteaga CL. ErbB2/neu kinase modulates cellular p27(Kip1) and cyclin D1 through multiple signaling pathways. Cancer Res 2001;61:6583-91.

20. Zhou BP, Liao Y, Xia W, Spohn B, Lee MH, Hung MC. Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. Nat Cell Biol 2001;3:245-52.

21. Algar E, Khromykh T, Smith S, Blackburn D, Bryson G, Smith P. A WT1 antisense oligonucleotide inhibits proliferation and induces apoptosis in myeloid leukaemia cell lines. Oncogene 1996;12:1005-14.

22. Yamagami T, Sugiyama H, Inoue K, Ogawa H, Tatekawa T, Hirata M, et al. Growth inhibition of human leukemic cells by WT1 (Wilms tumor gene) antisense oligodeoxynucleotides: implications for the involvement of WT1 in leukemogenesis. Blood 1996;87:2878-84.

23. Yamagami T, Ogawa H, Tamaki H, Oji Y, Soma T, Oka Y, et al. Suppression of Wilms' tumor gene (WT1) expression induces G2/M arrest in leukemic cells. Leuk Res 1998;22:383-84.

24. Smith SI, Down M, Boyd AW, Li CL. Expression of the Wilms' tumor suppressor gene, WT1, reduces the tumorigenicity of the leukemic cell line M1 in C.B-17 scid/scid mice. Cancer Res 2000;60:808-14.

25. Oji Y, Ogawa H, Tamaki H, Oka Y, Tsuboi A, Kim EH, et al. Expression of the Wilms' tumor gene WT1 in solid tumors and its involvement in tumor cell growth. Jpn J Cancer Res 1999;90:194-204.
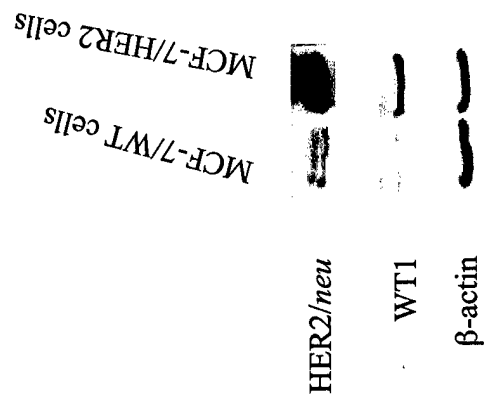
FIGURE LEGENDS

Figure 1. HER2/*neu* increases WT1 protein levels. (A) Western blot was used to determine the levels of WT1 protein in MCF-7 parental cells (MCF-7/WT) and MCF-7 cells transfected with *HER2/neu* (MCF-7/HER2) cells. (B) Western blot was used to determine the effects of HER2/*neu* inhibition by trastuzumab on WT1 expression. SKBr3 and BT-474 cells were incubated with 0.5 μM and 0.1 μM trastuzumab, respectively, for 5 days. Fifty μg of proteins were loaded on 10% SDS-PAGE. WT1 and phospho-HER2/*neu* (Tyr^{1268}) levels were determined. Grb2 protein was used as a loading control.

Figure 2. Inhibition of Akt activity leads to reduced WT1 protein levels in HER2/*neu*-overexpressing breast cancer cells. (A) SKBr3 and (B) BT-474 cells were plated at 0.75 x 10^5 cells/well and 1 x 10^5 cells/well, respectively, in 6-well plates in DMEM/F12 medium containing 5% FBS. After overnight attachment, SKBr3 and BT-474 cells were treated with 10 and 20 μM Akt inhibitor, respectively, for 4, 8, 24, and 72 h. WT1 and phospho-GSK-3β (Ser^9) levels were determined by western blot. Grb2 was used as loading control.
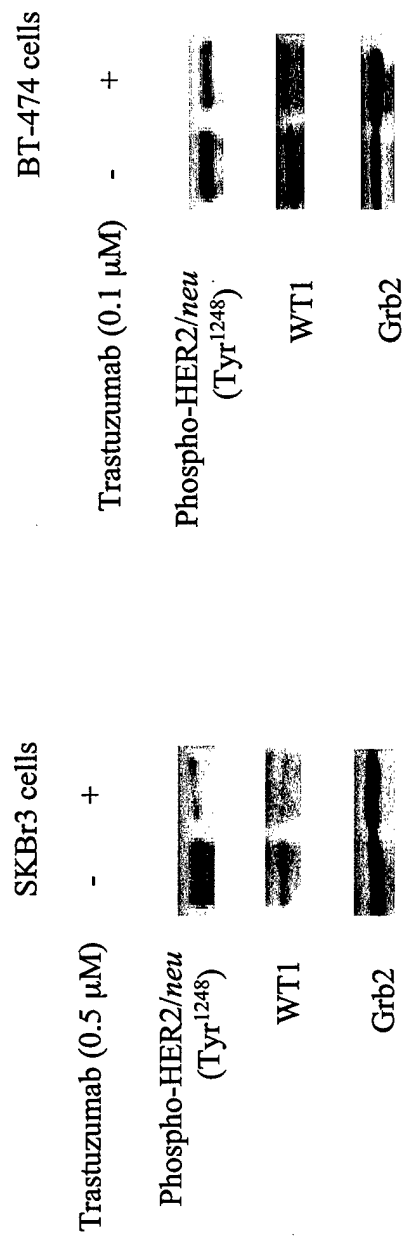
Figure 3. WT1 protein mediates proliferative and anti-apoptotic functions in BT-474 breast cancer cells. BT-474 cells were plated (0.75 x 10^5 cells/well) in 6 well plates in DMEM/F12 medium supplemented with 5% FBS. Cells were treated with 14 μM L-WT1 antisense or L-control oligos for 5 days. Approximately 1 x 10^6 cells were collected, and cell cycle and apoptosis were evaluated by flow cytometric analysis of propidium iodide staining.
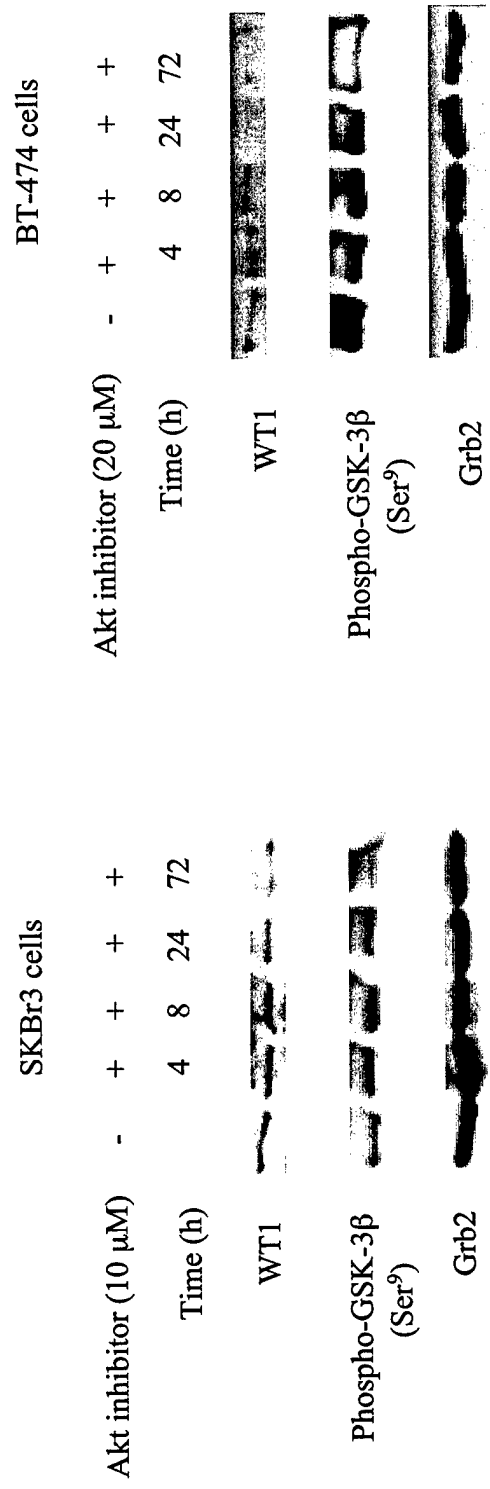
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Figure 4. WT1 protein regulates cyclin D1 and Bcl-2 expression in BT-474 cells. BT-474 cells were incubated with 14 μM L-WT1 antisense oligos, L-control oligos or empty liposomes for 5 days. (A) Western blot was used to determine the effects of WT1 downregulation on the levels of cyclin D1 and Bcl-2 proteins. (B) RT-PCR was used to determine the effects of WT1 downregulation on *cyclin* D1 and *Bcl-2* mRNA levels. β-actin was used as loading control.
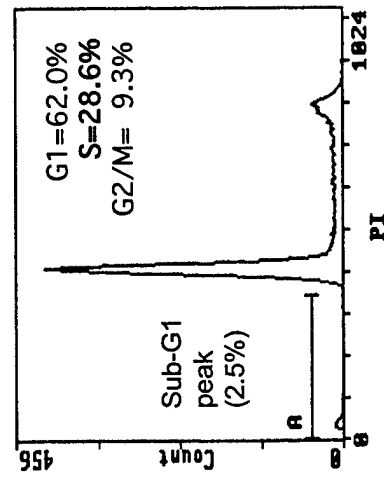
A

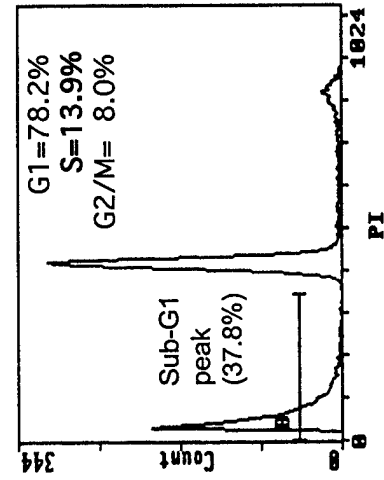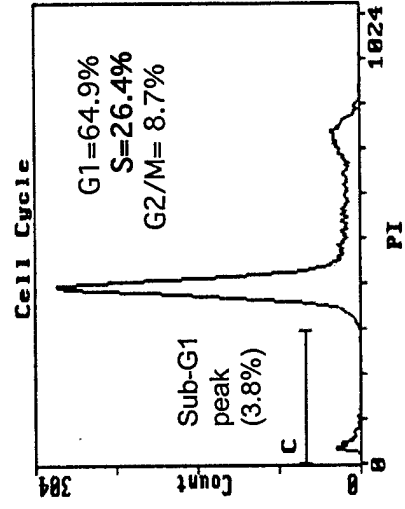MCF-7/HER2 cells
MCF-7/WT cells

HER2/neu

WT1

β-actin

B

SKBr3 cells

Trastuzumab (0.5 μM)   −   +

Phospho-HER2/neu
(Tyr^{1248})

WT1

Grb2

BT-474 cells

Trastuzumab (0.1 μM)   −   +

Phospho-HER2/neu
(Tyr^{1248})

WT1

Grb2

SKBr3 cells

Akt inhibitor (10 µM)   -   +   +   +   +

Time (h)   4   8   24   72

WT1

Phospho-GSK-3β
(Ser^9)

Grb2

BT-474 cells

Akt inhibitor (20 µM)   -   +   +   +   +

Time (h)   4   8   24   72

WT1

Phospho-GSK-3β
(Ser^9)

Grb2

Untreated

Sub-G1
peak
(2.5%)

G1=62.0%
S=28.6%
G2/M= 9.3%

L-WT1 antisense oligos
(14 μM)

Sub-G1
peak
(37.8%)

G1=78.2%
S=13.9%
G2/M= 8.0%

L-control oligos
(14 μM)

Cell Cycle

Sub-G1
peak
(3.8%)

G1=64.9%
S=26.4%
G2/M= 8.7%

A

WT1
Cyclin D1
Bcl-2
Grb2

Untreated
L-WT1 antisense oligos
L-control oligos
Empty liposomes

B

Cyclin D1
Bcl-2
β-actin

Untreated
L-WT1 antisense oligos
L-control oligos